





Food and Drug Administration 9200 Corporate Boulevard Rockville MD 20850

Russell K. Enns, Ph.D. Vice President of Regulatory Affairs Vysis, Inc. 3100 Woodcreek Drive Downers Grove, Illinois 60515

DEC 11 1998

Re: P980024

PathVysion™ HER-2 DNA Probe Kit

Filed: June 16, 1998

Amended: July 27, September 30, October 13, November 24 and

December 9, 1998

Dear Dr. Enns:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) has completed its review of your premarket approval application (PMA) for the PathVysion™ HER-2 DNA Probe Kit. The PathVysion™ HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2/neu gene via fluorescence in situ hybridization (FISH) in formalin-fixed paraffin-embedded human breast cancer tissue specimens. Results from the PathVysion Kit are intended for use as an adjunct to existing clinical and pathologic information currently used as prognostic factors in stage II, node-positive breast cancer patients. The PathVysion Kit is further indicated as an aid to predict disease-free and overall survival in patients with stage II, node positive breast cancer treated with adjuvant cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) chemotherapy. We are pleased to inform you that the PMA is approved subject to the conditions described below and in the "Conditions of Approval" (enclosed). You may begin commercial distribution of the device upon receipt of this letter.

The sale, distribution, and use of this device are restricted to prescription use in accordance with 21 CFR 801.109 within the meaning of section 520(e) of the Federal Food, Drug, and Cosmetic Act (the act) under the authority of section 515(d)(1)(B)(ii) of the act. FDA has also determined that, to ensure the safe and effective use of the device, the device is further restricted within the meaning of section 520(e) under the authority of section 515(d)(1)(B)(ii), (1) insofar as the labeling specify the requirements that apply to the training of laboratorians who may use the device as approved in this order and (2) insofar as the sale, distribution, and use must not violate sections 502(q) and (r) of the act.

Expiration dating for this device has been established and approved at twelve (12) months. This is to advise you that the protocol you used to establish this expiration dating is considered an approved protocol for the purpose of extending the expiration dating as provided by 21 CFR 814.39(a)(8).

Page 2 - Dr. Russell K. Enns

CDRH will notify the public of its decision to approve your PMA by making available a summary of the safety and effectiveness data upon which the approval is based. The information can be found on the FDA CDRH Internet HomePage located at http://www.fda.gov/cdrh/pmapage.html. Written requests for this information can also be made to the Dockets Management Branch, (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. The written request should include the PMA number or docket number. Within 30 days from the date that this information is placed on the Internet, any interested person may seek review of this decision by requesting an opportunity for administrative review, either through a hearing or review by an independent advisory committee, under section 515(q) of the Federal Food, Drug, and Cosmetic Act (the act).

Failure to comply with the conditions of approval invalidates this approval order. Commercial distribution of a device that is not in compliance with these conditions is a violation of the act.

You are reminded that, as soon as possible and before commercial distribution of your device, you must submit an amendment to this PMA submission with copies of all approved labeling in final printed form.

All required documents should be submitted in triplicate, unless otherwise specified, to the address below and should reference the above PMA number to facilitate processing.

PMA Document Mail Center (HFZ-401) Center for Devices and Radiological Health Food and Drug Administration 9200 Corporate Blvd. Rockville, Maryland 20850

If you have any questions concerning this approval order, please contact Peter E. Maxim, Ph.D. at (301) 594-1293.

Sincerely yours,

Kimber C. Richter, M.D.

Kimber C. Richter

Deputy Director for Clinical and Review Policy

Office of Device Evaluation Center for Devices and

Radiological Health

Enclosure

Issued: 3-4-98

CONDITIONS OF APPROVAL

APPROVED LABELING. As soon as possible, and before commercial distribution of your device, submit three copies of an amendment to this PMA submission with copies of all approved labeling in final printed form to the PMA Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration (FDA), 9200 Corporate Blvd., Rockville, Maryland 20850.

ADVERTISEMENT. No advertisement or other descriptive printed material issued by the applicant or private label distributor with respect to this device shall recommend or imply that the device may be used for any use that is not included in the FDA approved labeling for the device. If the FDA approval order has restricted the sale, distribution and use of the device to prescription use in accordance with 21 CFR 801.109 and specified that this restriction is being imposed in accordance with the provisions of section 520(e) of the act under the authority of section 515(d)(1)(B)(ii) of the act, all advertisements and other descriptive printed material issued by the applicant or distributor with respect to the device shall include a brief statement of the intended uses of the device and relevant warnings, precautions, side effects and contraindications.

PREMARKET APPROVAL APPLICATION (PMA) SUPPLEMENT. Before making any change affecting the safety or effectiveness of the device, submit a PMA supplement for review and approval by FDA unless the change is of a type for which a "Special PMA Supplement-Changes Being Effected" is permitted under 21 CFR 814.39(d) or an alternate submission is permitted in accordance with 21 CFR 814.39(e). A PMA supplement or alternate submission shall comply with applicable requirements under 21 CFR 814.39 of the final rule for Premarket Approval of Medical Devices.

All situations which require a PMA supplement cannot be briefly summarized, please consult the PMA regulation for further guidance. The guidance provided below is only for several key instances.

A PMA supplement must be submitted when unanticipated adverse effects, increases in the incidence of anticipated adverse effects, or device failures necessitate a labeling, manufacturing, or device modification.

A PMA supplement must be submitted if the device is to be modified and the modified device should be subjected to animal or laboratory or clinical testing designed to determine if the modified device remains safe and effective.

A "Special PMA Supplement - Changes Being Effected" is limited to the labeling, quality control and manufacturing process changes specified under 21 CFR 814.39(d)(2). It allows for the addition of, but not the replacement of previously approved, quality control specifications and test methods. These changes may be implemented before FDA approval upon acknowledgment by FDA that the submission is being processed as a "Special PMA Supplement - Changes Being Effected." This acknowledgment is in addition to that issued by the PMA Document Mail Center for all PMA supplements submitted. This procedure is not applicable to changes in device design, composition, specifications, circuitry, software or energy source.

Alternate submissions permitted under 21 CFR 814.39(e) apply to changes that otherwise require approval of a PMA supplement before implementation of the change and include the use of a 30-day PMA supplement or annual postapproval report. FDA must have previously indicated in an advisory opinion to the affected industry or in correspondence with the applicant that the alternate submission is permitted for the change. Before such can occur, FDA and the PMA applicant(s) involved must agree upon any needed testing protocol, test results, reporting format, information to be reported, and the alternate submission to be used.

POSTAPPROVAL REPORTS. Continued approval of this PMA is contingent upon the submission of postapproval reports required under 21 CFR 814.84 at intervals of 1 year from the date of approval of the original PMA. Postapproval reports for supplements approved under the original PMA, if applicable, are to be included in the next and subsequent annual reports for the original PMA unless specified otherwise in the approval order for the PMA supplement. Two copies identified as "Annual Report" and bearing the applicable PMA reference number are to be submitted to the PMA Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration, 9200 Corporate Blvd., Rockville, Maryland 20850. The postapproval report shall indicate the beginning and ending date of the period covered by the report and shall include the following information required by 21 CFR 814.84:

- (1) Identification of changes described in 21 CFR 814.39(a) and changes required to be reported to FDA under 21 CFR 814.39(b).
- (2) Bibliography and summary of the following information not previously submitted as part of the PMA and that is known to or reasonably should be known to the applicant:
 - (a)unpublished reports of data from any clinical investigations or nonclinical laboratory studies involving the device or related devices ("related" devices include devices which are the same or substantially similar to the applicant's device); and
 - (b) reports in the scientific literature concerning the device.

If, after reviewing the bibliography and summary, FDA concludes that agency review of one or more of the above reports is required, the applicant shall submit two copies of each identified report when so notified by FDA.

ADVERSE REACTION AND DEVICE DEFECT REPORTING. As provided by 21 CFR 814.82(a)(9), FDA has determined that in order to provide continued reasonable assurance of the safety and effectiveness of the device, the applicant shall submit 3 copies of a written report identified, as applicable, as an "Adverse Reaction Report" or "Device Defect Report" to the PMA Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration, 9200 Corporate Blvd., Rockville, Maryland 20850 within 10 days after the applicant receives or has knowledge of information concerning:

- (1)A mix-up of the device or its labeling with another article.
- (2) Any adverse reaction, side effect, injury, toxicity, or sensitivity reaction that is attributable to the device and
 - (a) has not been addressed by the device's labeling or

- (b) has been addressed by the device's labeling, but is occurring with unexpected severity or frequency.
- (3) Any significant chemical, physical or other change or deterioration in the device or any failure of the device to meet the specifications established in the approved PMA that could not cause or contribute to death or serious injury but are not correctable by adjustments or other maintenance procedures described in the approved labeling. The report shall include a discussion of the applicant's assessment of the change, deterioration or failure and any proposed or implemented corrective action by the applicant. When such events are correctable by adjustments or other maintenance procedures described in the approved labeling, all such events known to the applicant shall be included in the Annual Report described under "Postapproval Reports" above unless specified otherwise in the conditions of approval to this PMA. postapproval report shall appropriately categorize these events and include the number of reported and otherwise known instances of each category during the reporting period. Additional information regarding the events discussed above shall be submitted by the applicant when determined by FDA to be necessary to provide continued reasonable assurance of the safety and effectiveness of the device for its intended use.

REPORTING UNDER THE MEDICAL DEVICE REPORTING (MDR) REGULATION. The Medical Device Reporting (MDR) Regulation became effective on December 13, 1984. This regulation was replaced by the reporting requirements of the Safe Medical Devices Act of 1990 which became effective July 31, 1996 and requires that all manufacturers and importers of medical devices, including in vitro diagnostic devices, report to the FDA whenever they receive or otherwise become aware of information, from any source, that reasonably suggests that a device marketed by the manufacturer or importer:

- (1) May have caused or contributed to a death or serious injury; or
- (2) Has malfunctioned and such device or similar device marketed by the manufacturer or importer would be likely to cause or contribute to a death or serious injury if the malfunction were to recur.

The same events subject to reporting under the MDR Regulation may also be subject to the above "Adverse Reaction and Device Defect Reporting" requirements in the "Conditions of Approval" for this PMA. FDA has determined that such duplicative reporting is unnecessary. Whenever an event involving a device is subject to reporting under both the MDR Regulation and the "Conditions of Approval" for a PMA, the manufacturer shall submit the appropriate reports required by the MDR Requlation within the time frames as identified in 21 CFR 803.10(c) using FDA Form 3500A, i.e., 30 days after becoming aware of a reportable death, serious injury, or malfunction as described in 21 CFR 803.50 and 21 CFR 803.52 and 5 days after becoming aware that a reportable MDR event requires remedial action to prevent an unreasonable risk of substantial harm to the public health. The manufacturer is responsible for submitting a baseline report on FDA Form 3417 for a device when the device model is first reported under 21 CFR 803.50. This baseline report is to include the PMA reference number. Any written report and its envelope is to be specifically identified, e.g., "Manufacturer Report," "5-Day Report," "Baseline Report," etc.

Any written report is to be submitted to:

Food and Drug Administration Center for Devices and Radiological Health Medical Device Reporting PO Box 3002 Rockville, Maryland 20847-3002

Copies of the MDR Regulation (FOD # 336&1336) and FDA publications entitled "An Overview of the Medical Device Reporting Regulation" (FOD # 509) and "Medical Device Reporting for Manufacturers" (FOD #987) are available on the CDRH WWW Home Page. They are also available through CDRH's Fact-On-Demand (F-O-D) at 800-899-0381. Written requests for information can be made by sending a facsimile to CDRH's Division of Small Manufacturers Assistance (DSMA) at 301-443-8818.



SUMMARY OF SAFETY AND EFFECTIVENESS DATA

General Information I.

Device Generic Name:

Device for Detection of HER-2/neu Gene

Amplification in Human Breast Tissue

Device Trade Name:

PathVysion™ HER-2 DNA Probe Kit

Applicant's Name and

Address:

Vysis, Inc.

3100 Woodcreek Drive Downers Grove, IL 60515

Premarket Approval Application P980024

(PMA) Number:

Date of Panel Recommendation: November 9, 1998

Date of Notice of Approval

to the Applicant:

DEC 1 1 1999

II. **Indications for Use**

The PathVysion™ HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2/neu gene via fluorescence in situ hybridization (FISH) in formalin-fixed paraffin-embedded human breast cancer tissue specimens. Results from the PathVysion Kit are intended for use as an adjunct to existing clinical and pathologic information currently used as prognostic factors in stage II, node-positive breast cancer patients. The PathVysion Kit is further indicated as an aid to predict disease-free and overall survival in patients with stage II, node positive breast cancer treated with adjuvant cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) chemotherapy.

Warning:

The Vysis PathVysion Kit is not intended for use to screen for or diagnose breast cancer. It is intended to be used as an adjunct to other prognostic factors currently used to predict disease-free and overall survival in stage II, node-positive breast cancer patients. In making decisions regarding adjuvant CAF treatment, all other available clinical information should also be taken into consideration, such as tumor size, number of involved lymph nodes, and steroid receptor status. No treatment decision for stage II, node-positive breast cancer patients should be based on HER-2/neu gene amplification status alone.

The potential risks associated with misuse of the assay, or misinterpretation of the test results would be to assign patients to receive an adjuvant therapy regimen which is either too low for optimum effectiveness, or higher than necessary, potentially exposing the patient to serious side effects and, in rare cases, death. Selected patients with breast cancers shown to lack amplification of HER-2/neu, may still benefit from CAF adjuvant therapy on the basis of other prognostic factors which predict poor outcome. Conversely, selected patients with breast cancers shown to contain gene amplification may not be candidates for CAF therapy because of pre-existing or intercurrent medical illnesses. The dose and schedule of cyclophosamide, doxorubicin, and 5-fluorouracil in the CAF regimen have not been standardized.

Vysis will provide training in specimen preparation, assay procedure, and interpretation of FISH testing of the HER-2 gene for inexperienced users.

Background

Among all cancers in the U.S., breast cancer is expected to be the most common cancer (32% or 182,000 cases) in women and to be the second most common cause of death from cancer (18% or 46,000 cases) in 1995 [1]. After surgery, breast cancers with positive axillary nodes, which account for 30% of all breast cancers [2], are associated with a shorter disease-free survival [3,4] and a shorter overall survival [5] than node negative breast cancers. It has been generally accepted that patients with breast cancer and positive axillary nodes at diagnosis, should be offered adjuvant systemic treatment.

Amplification or overexpression of the HER-2/neu gene has been shown to be an indicator of poor prognosis in node-positive breast cancer [6-10]. In one study, the prognostic value of HER-2/neu appears to be stronger among patients treated with chemotherapy [7]. However, in predicting disease-free and overall survival in individual patients, other established prognostic factors such as tumor size, number of positive lymph nodes, and steroid receptor status must also be taken into consideration.

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The fluorescence *in situ* hybridization (FISH) technique has been used to detect HER-2/neu gene amplification in human breast carcinoma cell lines in both interphase and metaphse cells [11-14]. FISH appears to be an alternative technique capable of overcoming many of the inherent technical and interpretative limitations of other techniques, such as immunohistochemistry [15]. For quantification of HER-2/neu gene amplification, FISH assesses not only the level of HER-2/neu gene amplification directly in the tumor cells while retaining the characteristic morphology of the tissue studied, but also the spatial distribution of oncogene copies in individual uncultured primary breast carcinomas.

The LSI HER-2/neu DNA probe is a 190 Kb SpectrumOrange directly labeled fluorescent DNA probe specific for the HER-2/neu gene locus (17q11.2-q12). The CEP 17 DNA probe is a 5.4 Kb SpectrumGreen directly labeled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17q11.2-q11.1). The probes are pre-mixed and pre-denatured in hybridization buffer for ease of use. The assay is rapid, non-radioactive, requires little tumor material, and is capable of detecting as few as 2 to 8 copies of the oncogene.

III. Device Description

The PathVysion Kit permits identification and quantification of HER-2/neu gene amplification and contains four principal component reagents: Locus Specific Identifier® (LSI) HER-2/neu & Chromosome Enumeration Probe (CEP) 17 probe mixture, DAPI (4,6 diamidino-2-phenylindole) counterstain, Nonidet P-40 (NP-40), and 20X sodium chlorine/sodium citrate (SSC).

The PathVysion DNA probes are fluorescently labeled nucleic acid probes for use in *in situ* hybridization assay in paraffin-embedded tissue sections fixed on slides. The LSI HER-2/neu probe contains DNA sequences specific for HER-2/neu human gene locus and hybridizes to region 17q11.2-q12 of human chromosome 17. CEP 17 contains alpha satellite DNA that hybridizes to the D17Z1 locus. The CEP 17 probe is used as a control to determine copy number for chromosome 17. By enumerating copy numbers of HER-2/neu and CEP 17 in dual colors, the ratio of their average copy numbers per cell can be calculated to determine the presence of amplified HER-2/neu.

PRECAUTIONS

Selection of tissue for PathVysion assay and interpretation of assay results should be performed by a pathologist. To identify target areas, H & E staining should be conducted on every 10th slide of the same tissue block. Other Warnings and Precautions for use of the device are stated in the product labeling.

IV. Alternative Practices and Procedures

There is another FISH device to measure gene amplification in breast tissue from patients with localized invasive tumor who are lymph node negative for which there is an approved PMA. Alternative procedures to detect gene product overexpression in human breast tissue include immunohistochemical (IHC), or polymerase chain reaction (PCR) techniques.

V. Marketing History

This product has not been previously marketed for clinical use.

VI. Potential Adverse Effects of the Device on Public Health

The potential risks associated with misuse of the assay, or misinterpretation of (false positive) test results are to assign patients to receive a more agressive adjuvant therapy regimen than needed, potentially exposing the patient to serious side effects and, in rare cases, death. Alternatively, a patient who might have benefited from more aggressive therapy, may be excluded from a treatment regimen, potentially resulting in a poor outcome (false negative results).

VII. Summary of Studies

A. Non-Clinical Studies

1. Analytical

a. Hybridization Efficiency

On the ProbeChek[™] quality control slides, the average percentage of cells with no hybridization signal was 0.0 to 2.0%. These slides were prepared from paraffin-embedded breast cancer cell lines, and represented the best case scenario for hybridization efficiency. Under these conditions, the hybridization efficiency was expected to be 98%, with < 2% cells having no signal for either probe.

b. Analytical Sensitivity

The analytical sensitivity of the PathVysion Kit probes was tested in the reproducibility study described in 2 below. In that study, the 1.0 - 1.2 HER-2/neu:CEP 17 ratio specimen was estimated with a mean of 1.05 (± 0.03), and the 1.6 - 2.0 HER-2/neu:CEP 17 ratio specimen was estimated with a mean of 1.81 (± 0.08). The upper 95% Confidence

Interval (CI) was 1.11 for the 1.0 - 1.2 ratio specimen and the lower 95% CI for the 1.6 - 2.0 specimen was 1.65. The limit of detection for the PathVysion Kit in interphase cells was estimated to be a ratio of 1.5.

c. Analytical Specificity

Locus specificity studies were performed with metaphase spreads according to standard Vysis QC protocols. A total of 254 metaphase spreads were examined sequentially by G-banding to identify chromosome 17, and the HER-2/neu gene locus, followed by FISH. No cross-hybridization to other chromosome loci was observed in any of the 254 cells examined; hybridization was limited to the intended target regions of the two probes.

Stringency studies were also performed, according to standard Vysis protocols, on paraffin embedded tissue specimens to determine the optimum denaturation time and temperature; hybridization time and temperature; post-hybridization wash time and temperature; and posthybridization wash buffer composition. For the denaturation step, three temperatures (65° C, 73° C, and 80° C) were tested for 2 minutes, 5 minutes, and 8 minutes each. The results showed no statistical difference in the overall rating among all denaturation temperatures and durations; all combinations passed the quality evaluation. Stringency of the hybridization step was tested in two parts; first, hybridizations were conducted at 5 different temperatures (27° C, 32° C, 37° C, 42° C, and 47° C) for 18 hours, then for 5 different durations (10 hr, 14 hr, 18 hr, 22 hr, and 26 hr) at the recommended temperature (37° C). Hybridization was significantly affected by both hybridization temperature and time, with hybridizations at 37° C for 18 hours showing the highest overall quality ratings.

The post-hybridization wash step was tested in a similar manner; first assays were conducted at 5 different temperatures (69° C, 71° C, 73° C, 76° C, and 80° C), then for different durations, ranging from 2 to 8 minutes at 73° C. Wash temperature was a significant factor, with 73° C resulting in the highest ratings. Wash times between 2 and 5 minutes all produced acceptable results, but increasing the wash time to 8 minutes significantly lowered the overall quality ratings in some samples.

The wash buffer composition was also analyzed to determine the effect on signal intensity and probe specificity. Increasing the salt concentration from 0.4X Sodium Chloride and Sodium Citrate (SSC) to 2X SSC increased the signal intensity, but did not appear to compromise the probe specificity. A wash buffer composition of 2X SSC / 0.3% NP-40 is recommended.

d. Methods Comparison:

Using Vysis DNA probes for HER-2/neu and CEP17, FISH was compared to Southern, Northern and Western blot analyses, as well as IHC analysis on frozen specimens, in 143 archival breast cancer tissue specimens [11]. FISH was found to have a positive agreement of 96.5% relative to the IHC method on frozen specimens, while Southern blot analysis had an agreement of 92.4% relative to IHC. The agreement of Vysis FISH and IHC on negative specimens was 100%. Furthermore, the first-attempt success rate was 99% (with one failure due to loss of the tissue from the slide) for FISH analysis with direct-labeled probe (Vysis, Inc.), compared to 83% for Southern blot, 82% for Northern blot, 92% for Western blot, and 80% for FISH analysis with an indirect-labeled probe.

e. Stability

Expiration dating for this device has been established at twelve (12) months. The protocol used to establish this expiration dating is considered an approved protocol for the purpose of extending the expiration dating as provided by 21 CFR 814.39(a)(8).

2. Reproducibility and Repeatability Studies

The repeatability of the FISH assay for HER-2/neu was determined on consecutive sections of normal and amplified breast tissue, as well as on different thicknesses of the same tissue. On 10 consecutive tissue sections from one normal breast tissue, the average ratio of HER-2/neu to CEP 17 copy number was 1.19 (S.D. = 0.05); the results are shown in Table 1.

Table 1
Average Number of Signals per Cell and Ratio of
HER-2/neu:CEP 17 Copy Number in Consecutive Sections
(with normal HER-2/neu)

	Section Number									
	1	2	3	4	5	6	7	8	9	10
HER-2	3.8	3.2	3.6	3.5	3.6	3.5	3.4	3.5	3.3	3.1
CEP 17	3.1	3.1	3.0	2.7	3.0	3.0	2.8	2.1	3.0	2.7
Ratio	1.2	1.2	1.2	1.3	1.2	1.2	1.2	1.6	1.1	1.1

On 10 consecutive tissue sections from one specimen with amplified HER-2/neu, the average ratio of HER-2/neu to CEP 17 copy number was 3.61 (S.D.= 0.50); the results are shown in Table 2.

Table 2
Average Number of Signals per Cell and Ratio of
HER-2/neu:CEP 17 Copy Number in Consecutive Sections
(with amplified HER-2/neu)

	1	Section Number								
	1	2	3	4	5	6	7	8	9	10
HER-2	4.7	4.9	5.9	4.5	3.6	4.6	4.6	4.8	4.5	4.2
CEP 17	1.2	1.3	1.3	1.3	1.3	1.3	1.4	1.3	1.4	1.3
Ratio	3.9	3.7	4.7	3.6	2.8	3.7	3.3	3.8	3.3	3.3

Similarly, on 8 consecutive normal tissue sections of different thickness (2-8 microns), the average ratio of HER-2/neu to CEP 17 copy number was 1.15 (S.D..= 0.16); the results are shown in Table 3. These results demonstrated an acceptable degree of reproducibility of the HER-2/neu FISH assay in tissue sections with thicknesses between 4 and 8 microns.

Table 3

Average Number of Signals per Cell and Ratio of HER-2/neu :CEP 17

Copy Number in Consecutive Sections of Different Thickness

	Thickness of Section (microns)							
	2	2	4	4	6	6	8	8
HER-2	2.3	2.4	2.4	2.7	2.7	2.8	2.6	3.3
CEP 17	1.7	1.8	2.3	2.5	2.7	2.7	2.5	3.2
Ratio	1.4	1.4	1.1	1.1	1.0	1.1	1.1	1.0

Reproducibility using Control Slides:

The PathVysion Kit reproducibility was assessed for inter-site, inter-lot, inter-day and inter-observer reproducibility. Study specimens consisted of formalin-fixed, paraffin-embedded <u>human breast tumor cell lines</u> with four different levels of HER-2/neu amplification. The four ratios of LSI HER-2/neu to CEP 17 were 1-1.2 (normal), 1.6-2.0, 3-5, and 7-11, as determined by FISH. The specimens were evaluated for the ratio of LSI HER-2/neu to CEP 17 according to the instructions for signal enumeration in the package insert. Using analysis of variance (ANOVA) statistical methods, no significant variations were observed in any of the inter-assay reproducibility parameters. The results of the statistical analysis for the inter-assay reproducibility studies are summarized in Tables 4-7.

Table 4
Site-to-Site Reproducibility

		o Site Repi		
Ratio of HER-2/neu	Statistics	Site #1	Site #2	Site #3
to CEP 17		1		
	Mean	1.08	1.01	1.07
1.0-1.2	S.D	0.03	0.04	0.07
	C.V.(%)	2.66	3.58	6.77
	n	8	8	8
	Mean	1.81	1.71	1.78
1.6-2.0	S.D.	0.05	0.05	0.19
	C.V.(%)	2.88	2.78	10.50
	n	8	8	8
	Mean	4.39	3.65	4.49
3.0-5.0	S.D.	0.22	0.18	0.79
	C.V.(%)	4.99	4.93	17.64
	n	8	8	8
	Mean	7.21	8.26	8.23
7.0-11	S.D.	0.15	0.83	0.87
	C.V.(%)	2.07	10.10	10.55
	n	8	8	8

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 5
Lot-to-Lot Reproducibility

	,	1	,	,	·
Ratio of HER-2/neu to CEP 17	Statistics	Lot #1	Lot #2	Lot #3	Lot #4
	Mean	1.05	1.07	1.02	1.04
1.0-1.2	S.D	0.07	0.06	0.03	0.05
1	C.V.(%)	6.48	6.06	3.21	4.87
	n	6	6	6	6
	Mean	1.78	1.77	1.77	1.75
1.6-2.0	S.D.	0.10	0.13	0.15	0.09
	C.V.(%)	5.65	7.49	8.54	5.07
	n	6	6	6	6
	Mean	4.08	3.92	4.57	4.14
3.0-5.0	S.D.	0.44	0.34	0.96	0.40
	C.V.(%)	10.78	8.74	20.92	9.56
	n	6	6	6	6
	Mean	7.67	7.72	7.89	8.33
7.0-11	S.D.	0.69	0.72	0.88	1.06
	C.V.(%)	8.97	9.36	11.16	12.68
l	n	6	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 6
Day-to-Day Reproducibility

Ratio of	Statistics	Assay Day	Assay Day	Assay Day	Assay Day
HER-2/neu	Statistics	#1	#2	#3	#4
1		#1	m2	#3	# -
to CEP 17					
	Mean	1.06	1.07	1.02	1.04
1.0-1.2	S.D	0.06	0.07	0.05	0.04
	C.V.(%)	5.65	6.61	4.58	4.03
	n	6	6	6	6
	Mean	1.76	1.77	1.77	1.77
1.6-2.0	S.D.	0.17	0.14	0.08	0.10
	C.V.(%)	9.62	7.99	4.31	5.65
	n	6	6	6	6
	Mean	4.24	4.48	4.10	3.89
3.0-5.0	S.D.	0.48	0.97	0.36	0.38
	C.V.(%)	11.25	21.56	8.89	9.71
	n	6	6	6	6
	Mean	7.91	8.01	7.72	7.97
7.0-11	S.D.	1.11	0.90	0.57	0.89
	C.V.(%)	13.99	11.22	7.39	11.20
=	n	6	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 7
Observer-to-Observer Reproducibility

Ratio of	Statistics	Observer	Observer
HER-2/neu		#1	#2
to CEP 17			
	Mean	1.06	1.04
1.0-1.2	S.D	0.07	0.03
	C.V.(%)	7.00	2.85
	n	12	12
	Mean	1.71	1.82
1.6-2.0	S.D.	0.10	0.11
	C.V.(%)	6.01	6.20
	n	12	12
	Mean	4.05	4.31
3.0-5.0	S.D.	0.44	0.73
	C.V.(%)	10.80	16.84
	n	12	12
	Mean	7.52	8.28
7.0-11	S.D.	0.49	0.95
	C.V.(%)	6.55	11.44
	n	12	12

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

3. Portability Study

A five-center, blinded, randomized, comparative study using formalin-fixed, paraffin-embedded <u>human breast cancer specimens</u> was conducted to assess assay portability. Study specimens consisted of formalin-fixed, paraffin-embedded human breast cancer tissue sections with varying levels of HER-2/neu gene amplification (one normal, two with low level amplification, and one with moderate level amplification).

The intra-assay variations for all four ratios of LSI HER-2/neu to CEP 17 were estimated and presented in Table 8.

Table 8
Ratios of LSI HER-2/neu to CEP 17

Ratio of HER-2/neu to CEP 17	Mean	Standard Deviation	C.V. (%)	N
1.0-1.2	1.04	0.07	6.73	15
2.1-2.8	2.46	0.27	10.9	15
			8	
2.5-3.5	3.06	0.28	9.15	15
5.0-7.0	5.63	0.30	5.33	15

Day-to-Day Reproducibility

Table 9 shows the mean observed ratios of LSI HER-2/neu to CEP 17 for the three assay days.

Table 9
Summary Statistics of LSI HER-2/neu to CEP 17 by Assay Day

Expected Ratio	Statistics	Assay Day #1	Assay Day #2	Assay Day #3	P-value
	Mean	1.01	1.05	1.04	0.6205
1.0-1.2	S.D.	0.08	0.10	0.05	0.6395
	C.V. (%)	7.92	9.52	4.81	
	n	5	5	5	
	Mean	2.53	2.42	2.42	0.7(02
2.1-2.8	S.D.	0.11	0.28	0.39	0.7623
	C.V. (%)	4.34	11.57	16.12	
	n	5	5	5	
	Mean	3.17	2.98	3.03	0.5015
2.5-3.5	S.D.	0.27	0.30	0.30	0.5815
	C.V. (%)	8.52	10.07	9.90]
	n	5	5	5	
	Mean	5.66	5.60	5.62	0.0650
5.0-7.0	S.D.	0.29	0.25	0.42	0.9652
	C.V. (%)	5.12	4.46	7.47	
	n	5	5	5	

Site-to-Site Reproducibility

Table 10 shows the mean observed ratios of LSI HER-2/neu to CEP 17 for the five study sites.

Table 10
Summary Statistics of LSI HER-2/neu to CEP 17 by Assay Day

Expected	Statistics	Site	Site	Site #3	Site#4	Site#	
Ratio		#1	#2			5	P-value
	Mean	1.00	1.15	1.01	1.04	0.98	
1.0-1.2	S.D.	0.03	0.06	0.06	0.02	0.02	0.0032
	C.V. (%)	3.00	5.22	5.94	1.92	2.04	•
	n	3	3	3	3	3	
2.1.2.0	Mean	2.39	2.45	2.55	2.73	2.65	0.4010
2.1-2.8	S.D.	0.15	0.24	0.46	0.08	0.20	0.4919
	C.V. (%)	6.28	9.80	18.04	2.93	7.55	
	n	3	3	3	3	3	
2.5.5	Mean	3.00	3.09	3.41	2.73	3.08	0.0060
2.5-3.5	S.D.	0.16	0.38	0.12	0.08	0.12	0.0269
	C.V. (%)	5.33	12.30	3.52	2.93	3.90	
	n	3	3	3	3	3	
- 0 - 0	Mean	5.42	5.19	5.89	5.73	5.91	0.0001
5.0-7.0	S.D.	0.07	0.21	0.07	0.08	0.05	< 0.0001
	C.V. (%)	1.29	4.05	1.19	1.40	0.85	
	n	3	3	3	3	3	

B. Clinical Study

The objectives of this study were to determine whether the amplification of HER-2/neu, as assessed by FISH with DNA probe, provided statistically significant and independent prognostic information pertaining to disease-free survival and overall survival in stage II node-positive patients receiving adjuvant therapy.

Subject Selection and Exclusion Criteria

Only patients who met all of the following inclusion criteria were included:

- 1. Patients with node positive stage II breast cancer receiving adjuvant therapy in CALGB protocol 8869.
- 2. Sufficient archival paraffin-embedded tissue available for FISH assay.
- 3. Complete information available on relapse, survival, as well as other relevant clinical data.

Patients not meeting the inclusion criteria as specified above were excluded from the study.

Vysis Protocol 302 investigated whether HER-2/neu gene amplification could be used to identify those patients more likely to benefit from high doses of chemotherapy. FISH assay with the PathVysion Kit was performed on a sample of 572 patients, randomly selected from those patients included in the CALGB 8869 study. Among these 572 patients, 45 were excluded due to FISH assay failures, and 3 were duplicate assays. This left 524 cases for inclusion in the analysis of clinical utility (92% of the specimens were evaluable by FISH assay).

Analysis of Clinical Investigation

The results of analysis with Cox proportional hazard model for disease-free survival using FISH measurement of HER-2/neu gene amplification showed a statistically significant (p = 0.033) interaction between HER-2/neu gene amplification and the cyclophosphamide, anthracycline (doxorubicin), and 5-fluorouracil (CAF) dose regimen. Similarly, the results of Cox proportional hazard model for overall survival also showed a statistically significant (p = 0.028) interaction between HER-2/neu gene amplification and the CAF dose regimen (see Table 11).

Table 11
Cox Proportional Hazard Model Showing Liklihood Ratio Tests for Disease-free Survival and Overall Survival

	Diseas	e-Free Sur	vival	Overall	Survival	
Source	DF	ChiSq	P value	DF	ChiSq	P value
		Cilisq	1 value		Cittoq	1 value
CAF	2	5.56	0.06	2	4.57	0.10
Square root: #positive nodes	1	72.87	0.0000	1	, 56.32	0.0000
Tumor> 2cm	1	13.77	0.0002	1	12.93	0.0003
PREMENOPAUSAL	1	1.96	0.16	1	0.10	0.76
HER-2 ratio	1	10.05	0.0015	1	10.52	0.0012
HER-2 ratio interaction of CAF dose	2	6.84	0.033	2	7.15	0.028

As expected from the significance tests for the HER-2/neu by CAF inter-action from the proportional hazards models, there was a significant dose-response effect of adjuvant chemotherapy with CAF in patients with HER-2/neu gene

20

amplification, but not in patients with no or minimal HER-2/neu amplification. Disease-free survival probabilities (Figure 1(a,b)) were comparable among the three dose groups of patients with HER-2/neu -negative tumors.

At 7 years post-randomization the estimated disease-free survival probabilities were 55%, 63%, and 61% for low (L), moderate (M), and high (H) dose, respectively. The dose effect is greater for HER-2/neu positive tumors, with disease-free survival at 7 years of 36%, 44%, and 66% for L, M, and H, respectively (Table 12).

Table 12: Disease-free Probabilities

D)ose	HER MAN	HIP)R Daga jposlike
Low	55%	36%
Moderate	63%	44%
High	61%	66%

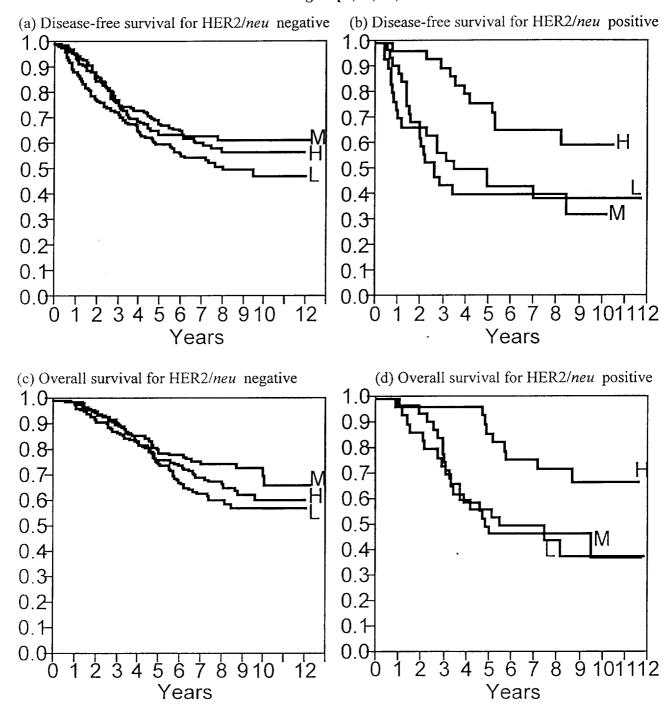
The corresponding figures for 7-year overall survival (Figure 1 (c,d)) have a similar relationship: 64%, 75%, and 70% for HER-2/neu negative and 48%, 50%, and 76%, again for L, M, and H, respectively (Table 13).

Table 13: Overall Survival Probabilities

Dose	HIBR 2 169 Hogshive	HER Drew positive
Low	64%	48%
Moderate	75%	50%
High	70%	76%

This association was found in both disease-free and overall survival, and was consistent with those observed with HER-2/neu expression by the immunohistochemistry method.

Figure 1
Disease-free (a, b) and overall (c, d) survival for patients with HER-2/neu negative (a, c) and positive (b, d) tumors for the three CAF dose groups, H, M, and L*



HER-2/neu positivity means HER/CEP 2. Sample sizes in (a, c) are 149, 136, and 148 (for H, M, and L) and in (b, d) are 30, 31, and 30. The significance levels for the HER2/neu by CAF interaction from the proportional hazards models (Tables 3.7 and 3.8) are 0.033 for disease-free survival—(a) vs. (b)—and 0.028 for overall survival—(c) vs. (d).

VIII. Conclusions Drawn from the Studies

A. Reproducibility Study

The results of this study demonstrated that an informative, quantitative analysis of different levels of HER-2/neu gene amplification can be performed using interphase nuclei. The precision of estimates for the ratio of LSI HER-2/neu to CEP 17 in the three amplified specimens was similar (3.04% to 5.53% coefficient of variation).

The analysis of ratio of HER-2/neu to CEP 17 demonstrated acceptable intraassay reproducibility and inter-assay reproducibility with respect to probe lot, assay day, institution, and observer. The reproducibility observed in this study indicated that the assay method and enumeration guide were clear and easy to follow. The between-observer correlation indicated that determination of HER-2/neu gene amplification with the HER-2/neu and CEP 17 assay required only one trained, experienced technologist.

In conclusion, the PathVysion Kit was of acceptable quality, easy to use, reproducible, and capable of providing reliable quantitative information for gene amplification in paraffin-embedded breast tissue specimens. For detection of HER-2/neu gene amplification, only one trained, experienced observer was needed for enumeration, and a simple procedure could be followed for quality assurance in practical applications. However, potential institutional differences should be recognized, and individual proficiency should be assessed prior to implementation of this test.

B. Clinical Study

FISH analysis with the PathVysion Kit on paraffin-embedded human breast tissue samples was reliable in determining the presence or absence of HER-2/neu amplification. FISH analysis of the study specimens showed that there was a statistically significant dose-response effect of adjuvant chemotherapy with CAF in patients with amplified HER-2/neu gene but not in patients with no or minimal HER-2/neu amplification. This association was found in both disease-free and overall survival, and was consistent with those observed with HER-2/neu expression by immunohistochemistry. In addition, this study found no correlation between HER-2/neu amplification, as assessed by FISH, and patient age, menopausal status, tumor size or the number of positive nodes. A statistically significant negative correlation was observed between HER-2/neu amplification and both estrogen (ER) and progesterone (PR) receptor status. This study also demonstrated that the recommended quality control methods were effective.

IX. Panel Recommendation

A public meeting of the FDA Immunology Devices Advisory Panel was conducted on November 9, 1998 to consider this PMA. The panel gave a recommendation of approvable with the following conditions:

- 1. Modify the Intended Use to read: "The PathVysion ™ HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2 gene via fluorescence in situ hybridization (FISH) in paraffin-embedded specimens from subjects with stage II, node positive breast cancer. Results from the PathVysion ™ HER-2 DNA Probe Kit are intended for use as a rapid assessment of the potential response to Adriamycin (doxorubicin) containing therapy. The testing will be performed in CLIA high complexity laboratories."
- 2. Prominently state in the package insert that a pathologist should be involved in both the selection of the slide area to be read and the assay's interpretation.
- 3. The package insert should state which specimens would be unacceptable for testing, e.g., insufficient specimen, necrotic tissue, and improper fixation or mishandled tissue blocks.
- 4. Following review by FDA, data obtained from the portability study that was conducted with patient samples should be included in the package insert.
- 5. User training should include tissue samples in addition to cell lines. The panel also recommended periodic proficiency testing with tissue samples should be encouraged.
- 6. Appropriate revisions to the labeling as recommended by FDA.

X. CDRH Action of the Application

CDRH issued an approval order for the applicant's PathVysion Kit on DEC 1 1 1998

The applicant's manufacturing and control facilities were inspected on September 14, 1998 and the facilities were found to be in compliance with the Good Manufacturing Practice Regulations (GMPs). The shelf-life of Vysis's PathVysionTM HER-2 DNA Probe Kit has been established at twleve (12) months when stored at 2-8° C.

XI. Approved Specifications

Directions for use: See attached labeling

Conditions of Approval: CDRH approval of this PMA is subject to full compliance with the conditions described in the approval order



XII. References

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Draft Document for PathVysion™ HER-2 DNA Probe Kit (LSI® HER-2/neu SpectrumOrange™ / CEP® 17 SpectrumGreen™) Package Insert

PathVysion™ HER-2 DNA Probe Kit
(LSI® HER-2/neu SpectrumOrange™ / CEP® 17 SpectrumGreen™)
(Part Number 32-161060)

Proprietary Name

PathVysion HER-2 DNA Probe Kit

Common or Usual Name

Fluorescence in situ hybridization (FISH) reagents

Intended Use

The PathVysion™ HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2/neu gene via fluorescence in situ hybridization (FISH) in formalin-fixed, paraffin-embedded human breast cancer tissue specimens. Results from the PathVysion Kit are intended for use as an adjunct to existing clinical and pathologic information currently used as prognostic factors in stage II, node-positive breast cancer patients. The PathVysion Kit is further indicated as an aid to predict disease-free and overall survival in patients with stage II, node positive breast cancer treated with adjuvant cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) chemotherapy.



Warning:

The Vysis PathVysion Kit is not intended for use to screen for or diagnose breast cancer. It is intended to be used as an adjunct to other prognostic factors currently used to predict disease-free and overall survival in stage II, node-positive breast cancer patients. In making decisions regarding adjuvant CAF treatment, all other available clinical information should also be taken into consideration, such as tumor size, number of involved lymph nodes, and steroid receptor status. No treatment decision for stage II, node-positive breast cancer patients should be based on HER-2/neu gene amplification status alone.

The potential risks associated with misuse of the assay, or misinterpretation of the test results would be to assign patients to receive an adjuvant therapy regimen which is either too low for optimum effectiveness, or higher than necessary, potentially exposing the patient to serious side effects and, in rare cases, death. Selected patients with breast cancers shown to lack amplification of HER-2/neu, may still benefit from CAF adjuvant therapy on the basis of other prognostic factors which predict poor outcome. Conversely, selected patients with breast cancers shown to contain gene amplification may not be candidates for CAF therapy because of pre-existing or intercurrent medical illnesses. The dose and schedule of cyclophosamide, doxorubicin, and 5-fluorouracil in the CAF regimen have not been standardized.

Vysis will provide training in specimen preparation, assay procedure, and interpretation of FISH testing of the HER-2 gene for inexperienced users.

Summary and Explanation

Among all cancers in the U.S., breast cancer is expected to be the most common cancer (32% / 182,000) in women and to be the second most common cause of death from cancer (18% / 46,000) in 1995 [1]. After surgery, breast cancers with positive axillary nodes, which account for 30% of all breast cancers [2], are associated with a shorter disease-free survival [3,4] and a shorter overall survival [5] than node negative breast cancers. It has been generally accepted that patients with breast cancer and positive axillary nodes at diagnosis, should be offered adjuvant systemic treatment.

Amplification or overexpression of the HER-2/neu gene has been shown to be an indicator of poor prognosis in node-positive breast cancer [6-10]. In one study, the prognostic value of HER-2/neu appears to be stronger among patients treated with chemotherapy [7]. However, in predicting disease-free and overall survival in individual patients, other established prognostic factors such as tumor size, number of positive lymph nodes, and steroid receptor status must also be taken into consideration.

The fluorescence *in situ* hybridization (FISH) technique has been used to detect HER-2/neu gene amplification in human breast carcinoma cell lines in both interphase and metaphase cells [11-14]. FISH appears to be an alternative technique capable of overcoming many of the inherent technical and interpretative limitations of other techniques, such as immunohistochemistry [15]. For quantification of HER-2/neu gene amplification, FISH assesses not only the level of HER-2/neu gene amplification directly in the tumor cells while retaining the characteristic morphology of the tissue studied, but also the spatial distribution of oncogene copies in individual uncultured primary breast carcinomas.

The LSI HER-2/neu DNA probe is a 190 Kb SpectrumOrange directly labeled fluorescent DNA probe specific for the HER-2/neu gene locus (17q11.2-q12). The CEP 17 DNA probe is a 5.4 Kb SpectrumGreen directly labeled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1). The probes are pre-mixed and pre-denatured in hybridization buffer for ease of use. The assay is rapid, non-radioactive, requires little tumor material, and is capable of detecting as few as 2 to 8 copies of the oncogene.

Reagents and Instruments

Materials Provided

This kit contains sufficient reagents to process approximately 20 assays. An assay is defined as one 22 mm x 22 mm target area.

1) LSI HER-2/neu SpectrumOrange (low copy no. E. coli vector) / CEP 17 SpectrumGreen DNA Probe (E. coli plasmid)

Vysis P.N.:

30-171060

Ouantity:

200 µL

Storage:

-20°C in the dark

Composition: SpectrumGreen fluorophore-labeled alpha satellite DNA probe

for chromosome 17, SpectrumOrange fluorophore-labeled DNA probe for the HER-2/neu gene locus, and blocking DNA, pre-

denatured in hybridization buffer.

2) DAPI Counterstain

Vysis P.N.:

30-804840

Quantity:

 $300 \, \mu L$

Storage:

-20°C in the dark

Composition: 1000 ng/mL DAPI (4,6-diamidino-2-phenylindole) in phenylenediamine dihydrochloride, glycerol, and buffer

3) NP-40

Vysis P.N.:

30-804820

Quantity:

4 mL (2 vials)

Storage:

-20 to 25°C

Composition: NP-40

4) 20X SSC salts

Vysis P.N.:

30-805850

Quantity:

66 g for up to 250 mL of 20X SSC solution

Storage:

-20 to 25°C

Composition: sodium chloride and sodium citrate

Note: Material Safety Data Sheets (MSDS) for all reagents provided in the kits are available upon request from the Vysis Technical Service Department.

Storage and Handling

Store the unopened PathVysion Kit as a unit at -20°C protected from light and humidity. The 20X SSC salts and NP-40 may be stored separately at room temperature. Store the control slides at 15-30°C in a sealed container with desiccant to protect them from humidity. Expiration dates for each of the unopened components are indicated on the individual component labels. These storage conditions apply to both opened and unopened components.

Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

Materials Required but Not Provided

Laboratory Reagents

ProbeChek HER-2/neu Normal Control Slides (Normal Signal Ratio)
 Vysis Cat. #30-805093

Formalin-fixed, paraffin-embedded, cultured human cell line (normal LSI HER-2/neu:CEP 17 ratio) applied to glass microscope slides

Ouantity: 5 slides

ProbeChek HER-2/neu Cutoff Control Slides (Weakly Amplified Signal Ratio)
 Vysis Cat. #30-805042

Formalin-fixed, paraffin-embedded, cultured human cell line (low level HER-2/neu amplification) applied to glass microscope slides

Ouantity: 5 slides

• Paraffin Pretreatment Reagent Kit (Vysis Cat. # 32-801200), which includes:

• Pretreatment Solution

NaSCN

Quantity: 5 x 50 mL

Protease

Pepsin (2500-3000 units/mg)

Quantity: 5 x 25 mg

Protease Buffer

NaCl solution, pH 2 Ouantity: 5 x 50 mL

 Wash Buffer 2X SSC, pH 7. Quantity: 2 x 250 mL

- Neutral buffered formalin solution (4% formaldehyde in PBS)
- Hemo-De clearing agent (Fisher Product No. 15-182-507A)
- Hematoxalin and eosin (H & E)
- Immersion oil appropriate fluorescence microscopy. Store at room temperature.
- Ultra-pure, formamide. Store at 4°C for up to one month from delivery (See manufacturer's recommendations for detailed information).
- Ethanol (100%). Store at room temperature.
- Concentrated (12N) HCl
- 1N NaOH
- Purified water (distilled or deionized or Milli-Q). Store at room temperature.
- Rubber cement
- Drierite and Nitrogen gas

Laboratory Equipment

- Precleaned silanized or positively charged glass microscope slides
- Slide warmer (45 50°C)
- 22 mm x 22 mm glass coverslips
- Microliter pipettor (1-10 µL) and sterile tips
- Polypropylene microcentrifuge tubes (0.5 or 1.5 mL)
- Timer
- Microtome
- Magnetic stirrer
- Vortex mixer
- Microcentrifuge
- Graduated cylinder
- Water baths $(37\pm1^{\circ}\text{C}, 72\pm1^{\circ}\text{C}, \text{ and } 80\pm1^{\circ}\text{C})$
- Protein-free water bath (40°C)
- Air incubators (37°C and 56°C)
- Diamond-tipped scribe
- Humidified hybridization chamber
- Forceps
- Disposable syringe (5 mL)
- Coplin jars (6) Suggested type: Wheaton Product No. 900620 vertical staining jar
- Fluorescent microscope equipped with recommended filters (see next section)
- pH meter and pH paper
- Calibrated thermometer
- Microscope slide box with lid
- 0.45 µm pore filtration unit

Microscope Equipment and Accessories

Microscope: An epi-illumination fluorescence microscope is required for viewing the hybridization results. If an existing fluorescence microscope is available, it should be checked to be sure it is operating properly to ensure optimum viewing of fluorescence in situ hybridization assay specimens. A microscope used with general DNA stains such as DAPI, Propidium Iodide, and quinacrine may not function adequately for FISH assays. Routine microscope cleaning and periodic "tune-ups" by the manufacturer's technical representative are advisable.

Note: Often, a presumed failure of reagents in an in situ assay may actually indicate that a malfunctioning or sub-optimal fluorescence microscope or incorrect filter set is being used to view a successful hybridization assay.

Excitation Light Source: A 100 watt mercury lamp with life maximum of about 200 hours is the recommended excitation source. Record the number of hours that the bulb has been used and replace the bulb before it exceeds the rated time. Ensure that the lamp is properly aligned.

<u>Objectives</u>: Use oil immersion fluorescence objectives with numeric apertures ≥ 0.75 when using a microscope with a 100 watt mercury lamp. A 40X objective, in conjunction with 10X eyepieces, is suitable for scanning. For FISH analysis, satisfactory results can be obtained with a 63X or 100X oil immersion achromat type objective.

<u>Immersion Oil:</u> The immersion oil used with oil immersion objectives should be one formulated for low auto fluorescence and specifically for use in fluorescence microscopy.

<u>Filters:</u> Multi-bandpass fluorescence microscope filter sets optimized for use with the CEP and LSI DNA probe kits are available from Vysis for most microscope models. The recommended filter sets for the PathVysion Kit are the DAPI/9-Orange dual bandpass, DAPI/Green dual bandpass and DAPI/Green/Orange triple bandpass. Hybridization of the LSI HER-2/neu and CEP 17 probes to their target regions is marked by orange and green fluorescence, respectively. All of the other DNA will fluoresce blue with the DAPI stain.

Preparation of Working Reagents

20X SSC (3M sodium chloride, 0.3M sodium citrate, pH 5.3)

To prepare 20X SSC pH 5.3, add together:

66 g 20X SSC 200 mL purified water 250 mL final volume

Mix thoroughly. Measure pH at room temperature with a pH meter. Adjust pH to 5.3 with concentrated HCl. Bring the total volume to 250 mL with purified water. Filter through a 0.45 μ m pore filtration unit. Store at room temperature for up to 6 months.

Denaturing Solution (70% formamide / 2X SSC, pH 7.0-8.0)

To prepare denaturing solution, add together:

49 mL formamide

7 mL 20X SSC, pH 5.3

14 mL purified water

70 mL final volume

Mix thoroughly. Measure pH at room temperature using a pH meter with glass pH electrode to verify that the pH is between 7.0 - 8.0. This solution can be used for up to one week. Check pH prior to each use. Store at 2 - 8°C in a tightly capped container when not in use.

Ethanol Solutions

Prepare v/v dilutions of 70%, 85%, and 100% using 100% ethanol and purified water. Dilutions may be used for one week unless evaporation occurs or the solution becomes diluted due to excessive use. Store at room temperature in tightly capped containers when not in use.

Post-Hybridization Wash Buffer (2X SSC/0.3% NP-40)

To prepare, add together:

100 mL 20X SSC, pH 5.3

847 mL Purified water

<u>3 mL</u> NP-40

1000 mL Final Volume

Mix thoroughly. Measure pH at room temperature using a pH meter. Adjust pH to 7.0 - 7.5 with 1N NaOH. Adjust volume to 1 liter with purified water. Filter through $0.45~\mu m$ pore filtration unit. Discard used solution at the end of each day. Store unused solution at room temperature for up to 6 months.

Warnings and Precautions

- 1. For In Vitro Diagnostic Use.
- 2. The PathVysion Kit is intended for use only on formalin-fixed, paraffin-embedded breast cancer tissue; it is not intended for use on fresh or non-breast cancer tissue.
- 3. All biological specimens should be treated as if capable of transmitting infectious agents. The control slides provided with this kit are manufactured from human cell lines that have been fixed in 10% formalin. Because it is often impossible to know which might be infectious, all human specimens and control slides should be treated with universal precautions. Guidelines for specimen handling are available from the U.S. Centers for Disease Control and Prevention [16].
- 4. Exposure of the specimens to acids, strong bases, or extreme heat, should be avoided. Such conditions are known to damage DNA and may result in FISH assay failure.
- 5. Failure to follow all procedures for slide denaturation, hybridization, and detection may cause unacceptable or erroneous results.
- 6. To identify target areas, H & E staining should be conducted on every 10th slide of the same tissue block.
- 7. Hybridization conditions may be adversely affected by the use of reagents other than those provided by Vysis, Inc.
- 8. Proper storage of kit components is essential to ensure the labeled shelf life. Assay results may be adversely effected by kit components stored under other conditions.
- 9. If stored at low temperatures, 20X SSC may crystallize. If the crystals cannot be redissolved at room temperature, the solution should be discarded.
- 10. If any other working reagents precipitate or become cloudy, they should be discarded and fresh solutions prepared.
- 11. The DAPI Counterstain contains DAPI (4,6-diamidino-2-phenylindole) and 1,4-phenylenediamine.
 - DAPI is a possible mutagen based on positive genotoxic effects. Avoid inhalation, ingestion, or contact with skin.
 - P-phenylenediamine is a known dermal sensitizer and a possible respiratory sensitizer. Avoid inhalation, ingestion, or contact with skin. Refer to MSDS for specific warnings.
- 12. Fluorophores are readily photobleached by exposure to light. To limit this degradation, handle all solutions containing fluorophores in reduced light. This includes all steps involved in handling the hybridized slide. Carry out all steps which do not require light for manipulation (incubation periods, washes, etc.) in the dark.
- 13. LSI HER-2/neu & CEP 17 DNA probe mixture contains formamide, a teratogen. Avoid contact with skin and mucous membranes.
- 14. Calibrated thermometers are required for measuring temperatures of solutions, water baths, and incubators.
- 15. Always verify the temperature of the pretreatment solution, denaturation solution and wash buffers prior to each use by measuring the temperature of the solution in the coplin jar with a calibrated thermometer.
- 16. All hazardous materials should be disposed of according to your local and state guidelines for hazardous disposal.

Specimen Processing and Slide Preparation

Specimen Collection and Processing

The PathVysion Kit is designed for use on formalin-fixed, paraffin-embedded tissue specimens. Tissue collections should be performed according to the laboratory's standard procedures. Selection of tissue for PathVysion assay should be performed by the pathologist. Exposure of the specimens to acids, strong bases, or extreme heat, should be avoided. Such conditions are known to damage DNA and may result in FISH assay failure.

Breast tissue should be prepared in sections between 4 and 6 microns thick. Formalin-fixed, paraffin-embedded tissue may be handled and stored according the laboratory's routine procedures. To ensure optimum results from the PathVysion Kit, these methods should be consistent for all specimens analyzed. To identify target areas, H & E staining should be conducted on every 10th slide of the same tissue block.

Tissue sections should be mounted on the positive side of an organosilane-coated slide in order to minimize detachment of the tissue from the slide during FISH assay. The PathVysion Kit contains reagents sufficient for approximately 20 assays; one assay for the PathVysion Kit is defined as a 22 mm x 22 mm area. Larger specimen sections will require more than 10 µL of probe per assay.

Slide Preparation from Formalin-Fixed, Paraffin-Embedded Tissue

The following method may be used for preparing slides from formalin-fixed, paraffin-embedded tissue specimens:

- 1. Cut 4-6 μm thick paraffin sections using a microtome.
- 2. Float the sections in a protein-free water bath at 40°C.
- 3. Mount the section on the positive side of an organosilane-coated slide.
- 4. Allow slides to air dry.
- 5. Bake slides overnight at 56°C.

Slide Pretreatment (Start processing ProbeChek control slides here)

Slides must be deparaffinized and the specimens fixed prior to assay with the PathVysion Kit. The package insert for the Vysis Paraffin Pretreatment Reagent Kit (Product No. 32-801200) contains detailed instructions. The following is a brief description of the procedure.

Deparaffinizing Slides

- Immerse slides in Hemo-De for 10 minutes at room temperature.
- Repeat twice using new Hemo-De each time.
- Dehydrate slides in 100% EtOH for 5 minutes at room temperature. Repeat.
- Air dry slides or place slides on a 45-50°C slide warmer.

Pretreating Slides

- Immerse slides in 0.2N HCl for 20 minutes.
- Immerse slides in purified water for 3 minutes.
- Immerse slides in Wash Buffer for 3 minutes.
- Immerse slides in Pretreatment Solution at 80°C for 30 minutes.
- Immerse slides in purified water for 1 minute.
- Immerse slides in Wash Buffer for 5 minutes. Repeat.

Protease Treatment

- Remove excess buffer by blotting edges of the slides on a paper towel.
- Immerse slides in Protease Solution at 37°C for 10 minutes.
- Immerse slides in Wash Buffer for 5 minutes. Repeat.
- Dry slides on a 45-50°C slide warmer for 2-5 minutes.

Fixing the Specimen

- Immerse the slides in neutral buffered formalin at room temperature for 10 minutes.
- Immerse the slides in wash buffer for 5 minutes. Repeat.
- Dry slides on a 45-50°C slide warmer for 2-5 minutes.
- Proceed with the PathVysion assay protocol.

Assay Procedure

Fluorescence In Situ Hybridization Procedure Summary

Denaturation of Specimen DNA

The timing for preparing the probe solutions should be carefully coordinated with denaturing the specimen DNA so that both will be ready for the hybridization step at the same time.

- 1. Prewarm the humidified hybridization chamber (an airtight container with a piece of damp blotting paper or paper towel approximately 1 in. x 3 in. taped to the side of the container) to 37°C by placing it in the 37°C incubator prior to slide preparation. Moisten the blotting paper or paper towel with water before each use of the hybridization chamber.
- 2. Verify that the pH of the denaturing solution is 7.0 8.0 at room temperature before use. Add denaturing solution to Coplin jar and place in a 72±1°C water bath for at least 30 minutes, or until the solution temperature reaches 72±1°C. Verify the solution temperature before use.
- 3. Mark the areas to be hybridized with a diamond-tipped scribe.
- 4. Denature the specimen DNA by immersing the prepared slides in the denaturing solution at 72±1°C (≤6 slides per jar) for 5 minutes. Do not denature more than 6 slides at one time per Coplin jar. Note: Verify the solution temperature before each use.
- 5. Using forceps, remove the slide(s) from the denaturing solution and immediately place into a 70% ethanol wash solution at room temperature. Agitate the slide to remove the formamide. Allow the slide(s) to stand in the ethanol wash for 1 minute.
- 6. Remove the slide(s) from 70% ethanol. Repeat step 5 with 85% ethanol, followed by 100% ethanol.
- 7. Drain the excess ethanol from the slide by touching the bottom edge of the slide to a blotter, and wipe the underside of the slide dry with a laboratory wipe.
- 8. Dry the slide(s) on a 45-50°C slide warmer for 2-5 minutes.

Probe Preparation

- 1. Allow the probe to warm to room temperature so that the viscosity decreases sufficiently to allow accurate pipetting.
- 2. Vortex to mix. Centrifuge each tube for 2-3 seconds in a bench-top microcentrifuge to bring the contents to the bottom of the tube. Gently vortex again to mix.

Hybridization

- 1. Apply 10 μL of probe mixture to target area of slide. Immediately, place a 22 mm x 22 mm glass coverslip over the probe and allow it to spread evenly under the coverslip. Air bubbles will interfere with hybridization and should be avoided. The remaining probe solution should be refrozen immediately after use.
- 2. Seal coverslip with rubber cement as follows: Draw the rubber cement into a 5 mL syringe. Eject a small amount of rubber cement around the periphery of the coverslip overlapping the coverslip and the slide, thereby forming a seal around the coverslip.
- 3. Place slides in the pre-warmed humidified hybridization chamber. Cover the chamber with a tight lid and incubate at 37°C overnight (14-18 hours).

Post-Hybridization Washes

- 1. Add post-hybridization wash buffer (2X SSC/0.3% NP-40) to a Coplin jar. Prewarm the post-hybridization wash buffer by placing the Coplin jar in the 72±1°C water bath for at least 30 minutes or until solution temperature has reached 72±1°C. Note: The temperature of the wash solution must return to 72±1°C before washing each batch.
- 2. Add post-hybridization wash buffer to a second Coplin jar and place at room temperature. Discard both wash solutions after 1 day of use.
- 3. Remove the rubber cement seal from the first slide by gently pulling up on the sealant with forceps.
- 4. Immerse slide(s) in post-hybridization wash buffer at room temperature and float off coverslip.

- 5. After coverslip has been carefully removed, remove excess liquid by wicking off the edge of the slide and immerse slide in post-hybridization wash buffer at 72±1°C for 2 minutes (≤6 slides/jar).
- 6. Remove each slide from the wash bath and air dry in the dark in an upright position. (A closed drawer or a shelf inside a closed cabinet is sufficient.)
- 7. Apply 10 µL of DAPI counterstain to the target area of the slide and apply a glass coverslip. Store the slide(s) in the dark prior to signal enumeration.

Slide Storage

Store hybridized slides (with coverslips) at -20°C in the dark. After removing from -20°C storage, allow slide(s) to reach room temperature prior to viewing using fluorescence microscopy.

Signal Enumeration

Assessing Slide Adequacy

Evaluate slide adequacy using the following criteria:

- Probe Signal Intensity: The signal should be bright, distinct, and easily
 evaluable. Signals should be in either bright, compact, oval shapes or
 stringy, diffuse, oval shapes.
- Background: The background should appear dark or black and relatively free of fluorescence particles or haziness.

If any of the above features are unsatisfactory, consult the troubleshooting guide (Table 2) and process a fresh slide.

Recognition of Target Signals

Use the prescribed filter (see pg. 8). Adjust the depth of the focus, and become familiar with the size and shape of the target signals and noise (debris). Enumerate hybridization signals only among tumor cells. Tumor cells in general are larger than normal cells, lymphocytes, and epithelial cells. Identify target areas by H & E stain on every 10th slide of the same tissue block. Identify these areas on the coverslip after the FISH assay is performed.

Selection of Optimum Viewing Area and Evaluable Nuclei

Use a 25X objective to view the hybridized area and locate the target of interest (tumor cells as identified by H & E stain). Avoid areas of necrosis and where the nuclear borders are ambiguous. Skip those nuclei with signals that require subjective judgment. Skip signals with weak intensity and non-specificity, or with noisy background. Skip nuclei with insufficient counterstain to determine the nuclear border. Enumerate only those nuclei with discrete signals.

Signal Enumeration

Using a 40X objective, scan several areas of tumor cells to account for possible heterogeneity. Select an area of good nuclei distribution; avoid areas of the target where hybridization signals are weak. Using a 63X or 100X objective, begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluable interphase cell according to the guidelines provided below and in Figure 1.

- Focus up and down to find all of the signals present in the nucleus.
- Count two signals that are the same size and separated by a distance equal or less than the diameter of the signal as one signal.
- Do not score nuclei with no signals or with signals of only one color. Score only those nuclei with one or more FISH signals of each color.
- Record counts in a two-way table such as that shown below.

Continue this process until 60 nuclei are enumerated and analyzed.

	CEP 17											
HER-2	0	1	2	3	4	5	6	7	8	9	10+	Total
0												
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11-15												
16-20			_									
21+												
Total												

Figure 1

Dual Color Signal Counting Guide

Key: O = green probe, CEP 17

• = orange probe, LSI HER-2/neu

1	000000000000000000000000000000000000000	Nuclei are overlapping and all areas of both of the nuclei are not visible but signals are not in overlapping area. Count as two orange and two green in each nucleus.
2	\$:•	Count as two orange signals and two green signals. One orange signal is diffuse.
3		Don't count. Nuclei are overlapping, all areas of nuclei are not visible and some signals are in overlapping area.
4	• 00	Count as two orange signals and two green signals. One orange signal is split.
5	® 0	Count as one orange signal and two green signals. One green signal is split and the orange signal is split.
6	•••	Count as two orange signals and one green signal.
7	0 •	Count as three orange signals and one green signal.
8	0 0	Count as four orange signals and two green signals.

Quality Control

Use of Control Slides

According to good clinical laboratory practices, control slides must be run concurrently with patient slides to monitor assay performance and to assess the accuracy of signal enumeration. Control slides should be used beginning with the Slide Pretreatment procedure. Controls should be run on each day of FISH testing and with each new kit lot. Vysis ProbeChek control slides are recommended.

Slide adequacy and signal enumeration should be assessed using the criteria described above in the signal enumeration section. The criteria for slide adequacy must be satisfied and the signal enumeration results should be within the specifications on the data sheets provided with the control slides for acceptable test performance.

If control slides fail to meet the slide acceptance criteria, the assay may not have been performed properly or the PathVysion Kit component(s) may have performed inadequately. A repeat analysis with fresh control slides and patient specimen slide(s) will be necessary. Consult the troubleshooting guide in Table 2 for probable causes and the actions needed to correct the problems.

If control slides meet the acceptance criteria but the enumeration values are outside the specified range, the enumeration may not have been performed correctly and an independent, repeat analysis of the same slide may be appropriate.

In no case should routine FISH test results be reported if assay controls fail. For clinical specimens, when interpretation of the hybridization signal is difficult and there is insufficient specimen sample for re-assay, the test is uninformative. If there are insufficient cells for analysis, the test is uninformative.

The PathVysion Kit contains five cutoff control slides (HER-2/neu weakly amplified signal ratio) and five negative control slides (HER-2/neu normal signal ratio). The negative control represents a specimen with a normal LSI HER-2/neu to CEP 17 signal ratio, and has a HER-2/neu to chromosome 17 copy number ratio of 1.0 to 1.2. The cutoff control represents weak HER-2/neu amplification (at or near the cutoff point) and has a HER-2/neu to chromosome 17 copy number ratio of 1.6 to 2.0. Additional cutoff (product number 30-805042) and negative (product number 30-805093) control slides are available from Vysis, Inc. Detailed descriptions of the acceptable ranges for the ratio of LSI HER-2/neu to CEP 17 are indicated on the specification data sheet that accompanies these slides.

The expected signal distribution for the normal and amplified signal ratio control slides are given in Table 1. If the results fall outside the expected range, consult the troubleshooting guide provided in Table 2.

Table 1
Signal Distribution for the ProbeChek HER-2/neu Normal and Amplified Signal Ratio Control Slides

		Normal	Ratio Cont						
			percentage	of cells with:					
	0 signals								
HER-2/neu	0.0-2.0	8.0-12.0	27.0-37.0	43.0-62.0	1.0-3.0	1.0-3.0			
CEP 17	0.0-2.0	13.0-17.0	22.0-32.0	43.0-63.0	1.0-3.0	1.0-3.0			
	Cutoff	(Weakly A	mplified R						
			percentage	of cells with:					
	0 signals	l signal	2 signals	3 signals	4 signals	>4 signals			
HER-2/neu	0.0-1.0	8.0-11.0	61.0-72.0	4.0-6.0	16.0-19.0	0.0-2.0			
CEP 17	0.0-1.0	65.0-75.0	25.0-29.0	0.0-1.0	0.0-3.0	0.0-1.0			

Signal distribution is based on hybridization using Vysis PathVysion HER-2/neu (Cat. no. 30-171060). Refer to the Certificate of Analysis for the actual counts on individual lot numbers.

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Table 2
Troubleshooting Guide

Problem	Probable Cause	Solution		
• No signal or weak signals	Inappropriate filter set used to view slides Microscope not functioning properly	Use recommended filters Call microscope manufacturer's technical representative		
	• Improper lamps (i.e. Xenon or Tungsten)	• Use a mercury lamp (100 watt recommended)		
	Mercury lamp too old	• Replace with a new lamp		
	Mercury lamp misaligned	• Realign lamp		
	Dirty and/or cracked collector lenses	Clean and replace lens		
	Dirty or broken mirror in lamp house	Clean or replace mirror		
	Hybridization conditions inappropriate	• Check temp of 37±1°C incubator • Increase hybridization time to at least 14 hours		
	Inappropriate post-hybridization wash temperature	• Check temp. of 72±1°C water bath		
	Air bubbles trapped under coverslip and	Apply coverslip by first touching the surface of		
	prevented probe access	the hybridization mixture		
	Insufficient amount of hybridization	• Increase amount of hybridization solution to 20		
	solution for section	μL per section		
	Inadequate protease digestion	• Check temp. of 37±1°C bath		
		• Check that pH of buffer is 2.0±0.2 • Increase digestion time, up to 60 min.		
	Section overfixed	• Eliminate fixation		
	- Section overtixed	• Optimal fixation time in preparation of paraffin		
		embedded slides is 24-48 hrs. Longer fixation		
		times will lead to progressive degradation of		
		morphology.		
	DNA loss (poor DAPI staining)	Check fixation conditions		
Variation of signal	Inherent in many tissue sections	Check DAPI staining. If DAPI staining in poor		
intensity across		area is good, then score slide. If DAPI staining		
tissue section		is poor in poor area, increase fixation time		
	• Probe unevenly distributed on slide due to	• Repeat hybridization on next adjacent section or		
	air bubbles under coverslip	same slide and make sure no air bubbles are		
		trapped under coverslip		
	Oversized section	• Increase volume of hybridization solution to 20		
		μL on large tissue sections		
 Noisy background 	Inadequate wash stringency	• Check pH of 7.2-7.5 wash buffer		
		• Check temperature of 72±1°C bath		
		• Provide gentle agitation during wash		
m' i .'	<u> </u>	• Increase wash time to 5 minutes		
• Tissue loss or tissue	• Tissue section underfixed	• Check fixation time/conditions		
morphology	(poor DAPI staining)	. The positively shored slides		
degraded	• Inappropriate slides used	• Use positively charged slides		
	• Improper slide baking	• Check temp. of 56°C oven • Check temp. of 80±1°C pretreatment		
	Over-pretreatment	• Decrease pretreatment time		
		Decrease preteatment time Decrease protease digestion time		
	Over-denaturation	• Check temp. of 72±1°C denaturation bath		
	J. C. G.	Decrease denaturation time		
	Tissue section overfixed	• Eliminate fixation step		
	(cell boundaries will be distinct)	Optimal fixation time in preparation of paraffin		
	(Soft Councilies will be distilled)	embedded slides is 24-48 hrs. Longer fixation		
		times will lead to progressive degradation of		
		morphology		
	Tissue section was torn removing	• Allow coverslip to soak off in wash buffer		
	I TESTE STEELS WAS TONE TONE TONE	Taran to totol p to come out in made outlet		

Contact the Vysis Technical Services Department at 800-553-7042 for further assistance



Interpretation of Results

The number of LSI HER-2/neu and CEP 17 signals per nucleus are recorded in a two-way table. Results on enumeration of 60 interphase nuclei from tumor cells per target are reported as the ratio of average HER-2/neu copy number to that of CEP 17. Our clinical study found that specimens with amplification showed a LSI HER-2/neu:CEP 17 signal ratio of ≥2.0; normal specimens showed a ratio of <2.0.

Results at or near the cutoff point (1.8 - 2.2) should be interpreted with caution. The specimen slide should be re-enumerated by another technician to verify the results. If still in doubt, the assay should be repeated with a fresh specimen slide. If the test results are not consistent with the clinical findings, a consultation between the pathologist and the treating physician is warranted.

Reasons to Repeat the Assay

The following are situations requiring repeat assays with fresh specimen slides and the appropriate control slides. Consult the troubleshooting guide (Table 2) for probable causes and the actions needed to correct specific problems.

- 1. If one or both of the control slides fail to meet the slide acceptance criteria, the specimen slide results are not reliable, and the assay must be repeated.
- 2. If there are fewer than 60 evaluable nuclei, the test is uninformative and the assay should be repeated.
- 3. If, upon assessing the slide quality as described in the Signal Enumeration section, any of the aspects (signal intensity, background, or cross-hybridization) are unsatisfactory, the assay must be repeated.



Limitations

- 1. The PathVysion Kit has been optimized only for identifying and quantifying chromosome 17 and the HER-2/neu gene in interphase nuclei from formalin-fixed, paraffin-embedded human breast tissue specimens. Other types of specimens or fixatives should not be used.
- The performance of the PathVysion Kit was validated using the procedures
 provided in this package insert only. Modifications to these procedures may alter
 the performance of the assay.
- Performance characteristics of the PathVysion Kit have been established only for node positive patients receiving the designated regimens of CAF. Performance with other treatment regimens has not been established.
- 4. The clinical interpretation of any test results should be evaluated within the context of the patient's medical history and other diagnostic laboratory test results.
- FISH assay results may not be informative if the specimen quality and/or specimen slide preparation is inadequate.
- 6. Technologists performing the FISH signal enumeration must be capable of visually distinguishing between the orange and green signals.

Expected Values

FISH interphase signal enumeration was performed on normal and amplified human breast tissue specimens to assess the expected ratio of LSI HER-2/neu to CEP 17.

FISH interphase analysis was performed on human breast tissue specimens from 433 subjects which were negative for HER-2/neu gene amplification by immunohistochemistry (IHC), to determine the expected values and to verify the cutoff point. Sixty nuclei were enumerated per specimen; the distribution of ratios of HER-2/neu to CEP 17 signals for these subjects is summarized in Table 3.

Table 3
Distribution of Ratio of HER-2/neu to CEP 17 Signals in Non-Amplified Breast Tissue Specimens

	Range				
Statistics	0.1-1.0	1.1-1.5	1.6-1.99		
mean	0.86	1.15	1.72		
S.D.	0.14	0.13	0.11		
n	185	226	22		

The cutoff point for assessing HER-2/neu gene amplification in breast tissue specimens was 2.0.

Ninety-one HER-2/neu amplified breast tissue specimens were also tested. The distribution of ratios of HER-2/neu to CEP 17 signals for these subjects is summarized in Table 4.

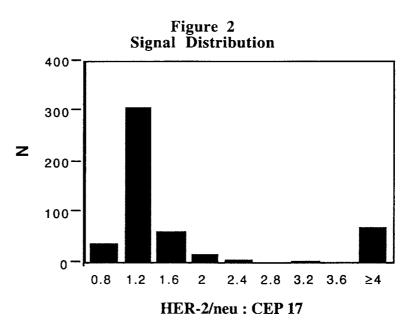
Table 4
Distribution of Ratio of HER-2/neu to CEP 17 Signals in Amplified
Breast Tissue Specimens

	Product Production							
	Range							
Statistics	2.0-5.0	5.1-10.0	>10.0					
mean	3.35	7.39	12.77					
S.D.	0.95	1.41	1.80					
n	33	42	16					



Establishment of Cut-off Point

In the pivotal study, the cutoff point for determining HER-2/neu gene amplification was determined to be 2.0, based on best fit analysis of clinical outcome of CAF treatment. Among the 433 non-amplified specimens, the largest ratio of LSI HER-2/neu to CEP 17 signals was 1.95, and among the 91 amplified samples, the smallest ratio of LSI HER-2/neu to CEP 17 signals was 2.0. This gap between the largest value among normal specimens and the smallest value among amplified specimens reduces the chance of misclassification, with 2.0 as the cutoff point. The distribution of the ratio of LSI HER-2/neu:CEP 17 in the 524 specimens from the study described above are shown in Figure 2.



Performance Characteristics

Analytical Sensitivity and Specificity

Hybridization Efficiency

On the ProbeChekTM quality control slides, the average percentage of cells with no hybridization signal was 0.0 to 2.0%. These slides are prepared from formalin-fixed, paraffin-embedded breast cancer cell lines, and represent the best case scenario for hybridization efficiency. Thus, under these conditions, the hybridization efficiency is expected to be 98%, with <2% cells having no signal for either probe.

Analytical Sensitivity

The analytical sensitivity of the PathVysion Kit probes was tested in the reproducibility study described below. In that study, the 1.0 - 1.2 HER-2/neu: CEP 17 ratio specimen was estimated with a mean of 1.05 (±0.03), and the 1.6 - 2.0 HER-2/neu: CEP 17 ratio specimen was estimated with a mean of 1.81 (±0.08). The upper 95% CI was 1.11 for the 1.0 - 1.2 ratio specimen and the lower 95% CI for the 1.6 - 2.0 specimen was 1.65. Thus, the limit of detection for the PathVysion Kit in interphase cells is estimated to be a ratio of 1.5.

Analytical Specificity

Locus specificity studies were performed with metaphase spreads according to standard Vysis QC protocols. A total of 254 metaphase spreads were examined sequentially by G-banding to identify chromosome 17, and the HER-2/neu gene locus, followed by FISH. No cross-hybridization to other chromosome loci was observed in any of the 254 cells examined; hybridization was limited to the intended target regions of the two probes.

Stringency studies were also performed, according to standard Vysis protocols, on formalin-fixed, paraffin-embedded tissue specimens to determine the optimum denaturation time and temperature; hybridization time and temperature; posthybridization wash time and temperature; and post-hybridization wash buffer composition. For the denaturation step, three temperatures (65°C, 73°C, and 80°C) were tested for 2 minutes, 5 minutes and 8 minutes each. The results showed no statistical difference in the overall rating among all denaturation temperatures and durations; all combinations passed the quality evaluation. Stringency of the hybridization step was tested in two parts; first, hybridizations were conducted at 5 different temperatures (27°C, 32°C, 37°C, 42°C, and 47°C) for 18 hours, then for 5 different durations (10 hr, 14 hr, 18 hr, 22 hr, and 26 hr) at the recommended temperature (37°C). Hybridization was significantly affected by both hybridization temperature and time, with hybridizations at 37°C for 18 hours showing the highest overall quality ratings. The post-hybridization wash step was tested in a similar manner; first assays were conducted at 5 different temperatures (69°C, 71°C, 73°C, 76°C, and 80°C), then for different durations, ranging from 2 to 8 minutes at 73°C. Wash temperature was a significant factor, with 73°C resulting in the highest ratings. Wash times between 2 and 5 minutes all produced acceptable results, but increasing the wash time to 8 minutes significantly lowered the overall quality ratings in some samples. The wash buffer composition was also analyzed to determine the effect on signal intensity and probe specificity. Increasing the salt concentration from 0.4X SSC to 2X SSC increased the signal intensity, but did not appear to compromise the probe specificity. Thus, a wash buffer composition of 2X SSC / 0.3% NP-40 is recommended.

Reproducibility

To assess the reproducibility of the HER-2/neu and CEP 17 assay, analyses for the ratio of HER-2/neu to CEP 17 were assessed for inter-site, inter-lot, inter-day, and inter-observer reproducibility on control slides with differing levels of HER-2/neu gene amplification. Four specimens consisting of formalin-fixed, paraffinembedded tissue from human breast tumor cell lines with normal (1.0-1.2) and amplified (1.6-2.0, 3-5, 7-11) ratios of HER-2/neu to CEP 17 were evaluated for HER-2/neu and CEP 17 according to the instructions for signal enumeration in the package insert. The overall hybridization success rate was 98.3% (118/120) on the first try. Hybridization of the two replacement slides was successful.

Using ANOVA, statistically significant variations were observed between observers, which reflects the subjectivity in signal interpretation and enumeration. No statistically significant variations were observed in any of the other study parameters. The mean, standard deviation, and percent CV of the observed ratios of HER-2/neu to CEP 17 are shown in Tables 5-8.



Table 5
Site-to-Site Reproducibility

Ratio of HER-2/neu to CEP 17	Statistics	Site #1	Site #2	Site #3
	Mean	1.08	1.01	1.07
1.0-1.2	S.D	0.03	0.04	0.07
	C.V.(%)	2.66	3.58	6.77
<u>L</u>	n	8	8	8
	Mean	1.81	1.71	1.78
1.6-2.0	S.D.	0.05	0.05	0.19
	C.V.(%)	2.88	2.78	10.50
	n	8	8	8
	Mean	4.39	3.65	4.49
3.0-5.0	S.D.	0.22	0.18	0.79
	C.V.(%)	4.99	4.93	17.64
	n	8	8	8
	Mean	7.21	8.26	8.23
7.0-11	S.D.	0.15	0.83	0.87
	C.V.(%)	2.07	10.10	10.55
	n	8	8	8

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 6
Lot-to-Lot Reproducibility

Datia of	1	o Bot Kep		<i>J</i>	
Ratio of HER-2/neu to CEP 17	Statistics	Lot #1	Lot #2	Lot #3	Lot #4
	Mean	1.05	1.07	1.02	1.04
1.0-1.2	S.D	0.07	0.06	0.03	0.05
	C.V.(%)	6.48	6.06	3.21	4.87
ļ 	n	6	6	6	6
	Mean	1.78	1.77	1.77	1.75
1.6-2.0	S.D.	0.10	0.13	0.15	0.09
	C.V.(%)	5.65	7.49	8.54	5.07
	n	6	6	6	6
	Mean	4.08	3.92	4.57	4.14
3.0-5.0	S.D.	0.44	0.34	0.96	0.40
	C.V.(%)	10.78	8.74	20.92	9.56
	n	6	6	6	6
	Mean	7.67	7.72	7.89	8.33
7.0-11	S.D.	0.69	0.72	0.88	1.06
	C.V.(%)	8.97	9.36	11.16	12.68
	n	6	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 7
Day-to-Day Reproducibility

Ratio of HER-2/neu	Statistics	Assay Day #1	Assay Day #2	Assay Day #3	Assay Day #4
to CEP 17		Day #1	Day #2	Day #3	Day #4
	Mean	1.06	1.07	1.02	1.04
1.0-1.2	S.D	0.06	0.07	0.05	0.04
	C.V.(%)	5.65	6.61	4.58	4.03
	n	6	6	6	6
	Mean	1.76	1.77	1.77	1.77
1.6-2.0	S.D.	0.17	0.14	0.08	0.10
	C.V.(%)	9.62	7.99	4.31	5.65
	n	6	6	6	6
	Mean	4.24	4.48	4.10	3.89
3.0-5.0	S.D.	0.48	0.97	0.36	0.38
	C.V.(%)	11.25	21.56	8.89	9.71
	n	6	6	6	6
	Mean	7.91	8.01	7.72	7.97
7.0-11	S.D.	1.11	0.90	0.57	0.89
	C.V.(%)	13.99	11.22	7.39	11.20
	n	6	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 8
Observer-to-Observer Reproducibility

SEED TO SEED TO THE PRODUCTION OF							
Ratio of	Statistics	Observer	Observer				
HER-2/neu		#1	#2				
to CEP 17							
	Mean	1.06	1.04				
1.0-1.2	S.D	0.07	0.03				
	C.V.(%)	7.00	2.85				
	n	12	12				
	Mean	1.71	1.82				
1.6-2.0	S.D.	0.10	0.11				
	C.V.(%)	6.01	6.20				
	n	12	12				
	Mean	4.05	4.31				
3.0-5.0	S.D.	0.44	0.73				
	C.V.(%)	10.80	16.84				
	n	12	12				
	Mean	7.52	8.28				
7.0-11	S.D.	0.49	0.95				
	C.V.(%)	6.55	11.44				
	n	12	12				

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Assay Portability

A five-center, blinded, randomized, comparative study using formalin-fixed, paraffin-embedded <u>human breast cancer specimens</u> was conducted to assess assay portability. Study specimens consisted of formalin-fixed, paraffin-embedded human breast cancer tissue sections with varying levels of HER-2/neu gene amplification. The specimens included one normal (no amplification), two with low level, and one with moderate level HER-2/neu gene amplification, as determined by FISH.

In this study, 100% of the specimens assayed yielded interpretable results on the first try.

Day-to-Day Reproducibility

The results of this study also demonstrated that the PathVysion assay is reproducible from day to day. Table 9 shows that the mean observed ratios of LSI HER-2/neu to CEP 17 for the three assay days varied within a narrow range, as evidenced by the low S.D.'s and C.V.'s.

Table 9
Summary Statistics of LSI HER-2/neu to CEP 17 by Assay Day

Expected Ratio	Statistics	Assay Day #1	Assay Day #2	Assay Day #3	P-value
1.0-1.2	Mean	1.01	1.05	1.04	0.6395
	S.D.	0.08	0.10	0.05	
	C.V.(%)	7.92	9.52	4.81	1
	n	5	5	5	
2.1-2.8	Mean	2.53	2.42	2.42	0.7623
	S.D.	0.11	0.28	0.39	
	C.V.(%)	4.34	11.57	16.12	
	n	5	5	5	
2.5-3.5	Mean	3.17	2.98	3.03	0.5815
	S.D.	0.27	0.30	0.30	
	C.V.(%)	8.52	10.07	9.90	
	n	5	5	5	
5.0-7.0	Mean	5.66	5.60	5.62	0.9652
	S.D.	0.29	0.25	0.42	
	C.V.(%)	5.12	4.46	7.47	
	n	5	5	5	



Site-to-Site Reproducibility

Table 10 shows that the mean observed ratios of LSI HER-2/neu to CEP 17 for the five study sites varied within a narrow range, as evidenced by the low S.D.'s and C.V.'s.

Table 10 Site-to-Site Reproducibility

Expected Ratio	Statistics	Site #1	Site #2	Site #3	Site #4	Site #5	P-value
1.0-1.2	Mean	1.00	1.15	1.01	1.04	0.98	0.0032
	S.D.	0.03	0.06	0.06	0.02	0.02	
	C.V.(%)	3.00	5.22	5.94	1.92	2.04	
	n	3	3	3	3	3	
2.1-2.8	Mean	2.39	2.45	2.55	2.26	2.65	0.4919
	S.D.	0.15	0.24	0.46	0.18	0.20	
	C.V.(%)	6.28	9.80	18.04	7.95	7.55	
	n	3	3	3	3	3	
2.5-3.5	Mean	3.00	3.09	3.41	2.73	3.08	0.0269
	S.D.	0.16	0.38	0.12	0.08	0.12	
	C.V.(%)	5.33	12.30	3.52	2.93	3.90	
	n	3	3	3	3	3	
5.0-7.0	Mean	5.42	5.19	5.89	5.73	5.91	< 0.0001
	S.D.	0.07	0.21	0.07	0.08	0.05	
	C.V.(%)	1.29	4.05	1.19	1.40	0.85	
	n	3	3	3	3	3	

The summary of assay variations for all five are presented in Table 11. The standard deviation (S.D.) and the coefficient of variation (C.V.) were small and relatively stable across all ratios of LSI HER-2/neu to CEP 17.

Table 11 Summary of Site-to-Site Reproducibility

Ratio of HER-2/neu to CEP 17	Mean	Standard Deviation	C.V. (%)	N
1.0-1.2	1.04	0.07	6.73	15
2.1-2.8	2.46	0.27	10.98	15
2.5-3.5	3.06	0.28	9.15	15
5.0-7.0	5.63	0.30	5.33	15

This study had a 100% (60/60) hybridization success rate, validating the ease of use of the PathVysion HER-2 Kit.

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Clinical Study

The interaction between HER-2/neu gene amplification and dose of CAF was evaluated in a retrospective analysis of a single randomized clinical trial, CALGB 8869. This was a large, prospective, randomized trial in stage II, node-positive breast cancer patients that evaluated three different doses of adjuvant CAF chemotherapy: a high dose (cyclophosphamide at 600 mg/m², doxorubicin at 60 mg/m², and 5-fluorouracil at 600 mg/m² for four cycles), a moderate dose (cyclophosphamide at 400 mg/m², doxorubicin at 40 mg/m², and 5-fluorouracil at 400 mg/m² for six cycles), or a low dose (cyclophosphamide at 300 mg/m², doxorubicin at 30 mg/m², and 5-fluorouracil at 300 mg/m² for four cycles). Doses were administered on a 28-day cycle.

Archived tissue specimens from 572 patients, randomly selected from the original study population, were included for analysis by FISH assay with DNA probe. The objectives of this study were to determine whether amplification of the HER-2/neu gene provides statistically significant and independent prognostic information pertaining to disease-free survival and overall survival in stage II, node positive breast cancer patients receiving adjuvant CAF therapy; and to explore the relationship between HER-2/neu gene amplification and clinical data, including such factors as tumor grade and steroid receptor status.

Among these 572 tumor specimens, 45 were excluded due to FISH assay failures, and 3 were duplicate assays. This left 524 cases for analysis. Using the Vysis PathVysion DNA Probe Kit, HER-2/neu gene amplification was defined as >2 (i.e., the ratio of average HER-2/neu to average CEP 17 signals with 60 nuclei counted). A total of 433 patient samples were found to be HER-2/neu-negative and 91 HER-2/neu-positive.

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The table below lists the several baseline characteristics of the 524 patients whose archived tumor specimens were selected for evaluation by this assay, as well as, details of the adjuvant treatments received on the original CALGB 8869 study.

Table 12
Comparison of Patient Characteristics at Baseline and Details of
Adjuvant Treatment

Aujuvant Treatment			
	HER-2/neu amplification*	No HER-2/neu amplification*	
	n=91	n=433	
Age			
<40	17.6	14.5	
40-50	39.6	40.0	
>50	42.9	48.5	
Premenopausal	46.2	39.5	
Peri/Postmenopausal	53.8	60.5	
Tumor size		00.0	
≤2 cm	31.9	37.2	
>2 - ≤5	57.1	58.4	
>5	9.9	3.9	
unknown	1	0.5	
Positive nodes			
≤3	59.3	55.9	
4-9	27.5	34.9	
≥10	13.2	9.2	
ER (+)	49.5	71.4	
PR(+)	35.2	61.7	
ER (+) or PR (+)	60.4	77.8	
CAF dose regimen received		7	
High	33.0	34.4	
Moderate	34.1	31.4	
Low	33.0	34.2	
*		1 37.4	

^{*}percent of patients

The results of analysis with Cox proportional hazard model for disease-free survival using FISH measurement of HER-2/neu gene amplification showed a statistically significant interaction between HER-2/neu gene amplification and the CAF dose regimen received (p=0.033, likelihood test, see Table 13). Similarly, the results of Cox proportional hazard model for overall survival also showed a statistically significant interaction between HER-2/neu gene amplification and the CAF dose regimen received (p=0.028, likelihood test, see Table 13)

Table 13
Cox Proportional Hazard Model Showing Likelihood-Ratio Tests for Disease-free and Overall Survival

Disease free and Overall Survival				
	Disease-Free Survival		Overall Survival	
Source	DF	ChiSq P value	DF	ChiSq P value
CAF	2	5.56 0.06	2	4.57 0.10
Square root: # positive nodes	1	72.87 0.0000	1	56.32 0.0000
Tumor > 2 cm	1	13.77 0.0002	1	12.93 0.0003
Premenopausal	1	1.96 0.16	1	0.10 0.76
HER-2 ratio	1	10.05 0.0015	1	10.52 0.0012
HER-2 ratio interaction of CAF dose	2	6.84 0.033	2	7.15 0.028

Disease-free survival probabilities (Table 14, Figure 3a) are comparable among the three dose groups of patients with HER-2/neu -negative tumors. For example, at 7 years post-randomization the estimated disease-free survival probabilities are 55%, 63%, and 61% for low (L), moderate (M), and high (H) CAF dose groups, respectively. The dose effect is greater for patients with HER-2/neu- positive tumors (Table 14, Figure 3b), with disease-free survival at 7 years of 36%, 44%, and 66% for L, M, and H CAF dose groups, respectively. The corresponding figures for overall survival at 7 years (Table 15, Figure 3c) have a similar relationship: 64%, 75%, and 70% for patients with HER-2/neu -negative tumors, and 48%, 50%, and 76% for patients with HER-2/neu- positive tumors, again for L, M, and H CAF dose groups, respectively (Table 15, Figure 3d).

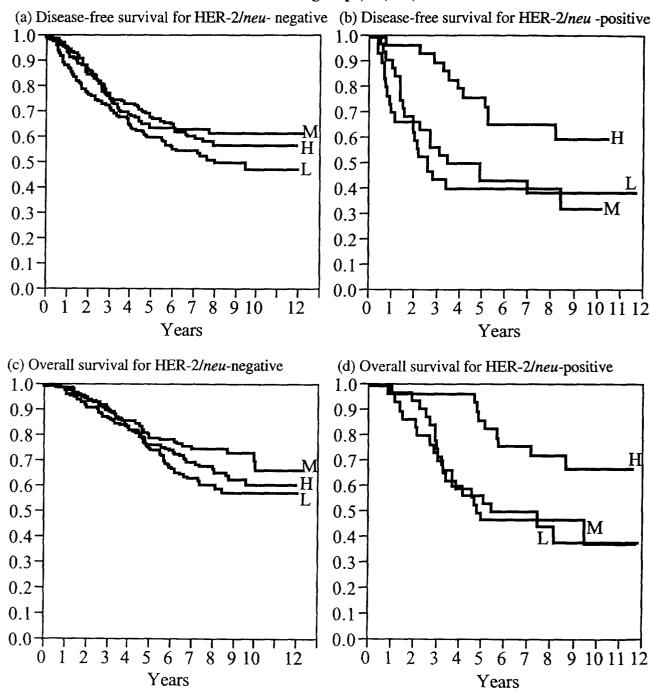
Table 14
Disease-free Survival Probabilities

Disease-free Survivar Trobabilities			
CAF Dose	HER-2/neu negative	HER-2/neu positive	
Low	55%	36%	
Moderate	63%	44%	
High	61%	66%	

Table 15 Overall Survival Probabilities

CAF Dose	HER-2/neu negative	HER-2/neu positive
Low	64%	48%
Moderate	75%	50%
High	70%	76%

Figure 3
Disease-free (a, b) and overall (c, d) survival for patients with HER-2/neu-negative (a, c) and positive (b, d) tumors for the three CAF dose groups, H, M, and L*



*HER-2/neu positivity means HER/CEP \geq 2. Sample sizes in (a, c) are 149, 136, and 148 (for H, M, and L) and in (b, d) are 30, 31, and 30. The significance levels for the HER-2/neu by CAF interaction from the proportional hazards models (Tables 10 and 11) are 0.033 for disease-free survival—(a) vs. (b)—and 0.028 for overall survival—(c) vs. (d).

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FISH analysis of the study specimens showed that there was a significant dose-response effect of adjuvant chemotherapy with CAF in patients with HER-2/neu gene amplification, but not in patients with no or minimal HER-2/neu amplification. This association was found in both disease-free and overall survival. In addition, this study found no correlation between HER-2/neu copy number, as assessed by FISH, and patient age, menopausal status, tumor size or the number of positive nodes. A statistically significant negative correlation was observed between HER-2/neu copy number and both estrogen (ER) and progesterone (PR) receptor status.

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